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### Research Article

We investigated the lobular localization and molecular level of expression of cholesterol 7 alpha-hydroxylase and sterol 27-hydroxylase, two key enzymes in bile acid synthesis, in isolated periportal and pericentral hepatocytes and by in situ hybridization of rat liver. Enzyme activity, mRNA, and gene transcription of cholesterol 7 alpha-hydroxylase were predominant in pericentral hepatocytes of control rats, being 7.9-, 9.9-, and 4.4-fold higher than in periportal hepatocytes, respectively. Similar localization was found for sterol 27-hydroxylase: 2.9-, 2.5-, and 1.7-fold higher enzyme activity, mRNA, and gene transcription, respectively, was found in pericentral hepatocytes. Interruption of the enterohepatic circulation with colestid resulted in upregulation of these parameters for both enzymes, as a consequence of stimulated gene expression mainly in the periportal zone. In contrast, mRNA levels and gene transcription of 3-hydroxy-3-methylglutaryl CoA reductase showed opposite lobular distribution. Selective periportal expression for the latter was enhanced, but remained local, after colestid treatment. In situ hybridization showed unambiguously that cholesterol 7 alpha-hydroxylase mRNA is localized exclusively in the pericentral zone and that sterol 27-hydroxylase mRNA is expressed preferentially in the pericentral region, though less pronounced. Administration of colestid led to expression of both genes within a larger area of the liver lobulus. In conclusion, we suggest that cholesterol 7 alpha-hydroxylase and sterol 27-hydroxylase are coordinately regulated by the bile acid gradient over the lobulus, resulting [...]

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# Heterogeneous Expression of Cholesterol 7 $\alpha$ -Hydroxylase and Sterol 27-Hydroxylase Genes in the Rat Liver Lobulus

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## Abstract

We investigated the lobular localization and molecular level of expression of cholesterol 7 $\alpha$ -hydroxylase and sterol 27-hydroxylase, two key enzymes in bile acid synthesis, in isolated periportal and pericentral hepatocytes and by *in situ* hybridization of rat liver. Enzyme activity, mRNA, and gene transcription of cholesterol 7 $\alpha$ -hydroxylase were predominant in pericentral hepatocytes of control rats, being 7.9-, 9.9-, and 4.4-fold higher than in periportal hepatocytes, respectively. Similar localization was found for sterol 27-hydroxylase: 2.9-, 2.5-, and 1.7-fold higher enzyme activity, mRNA, and gene transcription, respectively, was found in pericentral hepatocytes. Interruption of the enterohepatic circulation with colestid resulted in upregulation of these parameters for both enzymes, as a consequence of stimulated gene expression mainly in the periportal zone. In contrast, mRNA levels and gene transcription of 3-hydroxy-3-methylglutaryl CoA reductase showed opposite lobular distribution. Selective periportal expression for the latter was enhanced, but remained local, after colestid treatment. *In situ* hybridization showed unambiguously that cholesterol 7 $\alpha$ -hydroxylase mRNA is localized exclusively in the pericentral zone and that sterol 27-hydroxylase mRNA is expressed preferentially in the pericentral region, though less pronounced. Administration of colestid led to expression of both genes within a larger area of the liver lobulus. In conclusion, we suggest that cholesterol 7 $\alpha$ -hydroxylase and sterol 27-hydroxylase are coordinately regulated by the bile acid gradient over the lobulus, resulting in predominant expression in the pericentral zone. Opposite lobular localization of cholesterol and bile acid synthesis provides an alternative view to interregulation of these metabolic pathways. (*J. Clin. Invest.* 1995. 95:1235–1243.) Key words: lobular heterogeneity • bile acid synthetic enzyme • mRNA • transcriptional activity • *in situ* hybridization

## Introduction

The liver plays an important role in the homeostatic maintenance of a large number of nutrients in the blood, such as

carbohydrates, amino acids, and lipids, and is the main site of their intermediary metabolism. It has become increasingly clear that not all hepatocytes contribute equally in this task. In contrast, contribution of hepatocytes to uptake, storage, interconversion, and release of various compounds shows a large degree of heterogeneity along the portocentral axis; in some cases only a few cells are involved in a given function (1–4). The concept of “metabolic zonation” dictates that the heterogeneous expression of enzymes in the liver lobulus (or acinus) is a major determinant for the proper execution and regulation of various liver functions (5, 6). Opposite metabolic pathways like gluconeogenesis and glycolysis are performed simultaneously by hepatocytes in periportal and pericentral zones, respectively, and are separately localized within the liver (1, 3). Importantly, the distribution may be dynamic under different physiological and pathological conditions, in the sense that the liver may adapt to certain requirements by changes in distribution patterns. Liver cell heterogeneity thus provides the basis for effective regulation and adaptation to different metabolic states.

It has previously been shown that cholesterol synthesis is localized predominantly in the periportal hepatocytes, as judged from the positive immunohistochemical staining for 3-hydroxy-3-methylglutaryl CoA (HMG-CoA)<sup>1</sup> synthase and HMG-CoA reductase protein in only 20% of the periportal cell fraction (7, 8). Excretion of cholesterol into bile, either as free cholesterol or after its conversion into bile acids, is the predominant pathway for elimination of cholesterol from circulation in mammals (9, 10). The rate of bile acid formation is therefore considered an important determinant for cholesterol homeostasis. On the other hand, bile acid synthesis and the major key enzyme in routing of cholesterol to bile acids, cholesterol 7 $\alpha$ -hydroxylase, are localized mainly pericentrally (11, 12). Opposite lobular localization of cholesterol synthetic and metabolic pathways poses the interesting question of how the two are interregulated to achieve homeostasis.

Another aspect of bile acid synthesis concerns the different pathways of bile acid formation. According to current views, the initial and rate-determining step in routing of cholesterol to bile acids is catalyzed by cholesterol 7 $\alpha$ -hydroxylase (9). However, accumulating evidence has led to the suggestion that an alternative pathway exists, involving initial 27-hydroxylation of cholesterol via sterol 27-hydroxylase (13). Based on both *in vivo* studies in humans (13) and studies using cultured human and rat hepatocytes (14), it was concluded that this alternative pathway may contribute considerably to total bile acid synthesis (13, 14). Hitherto, lobular expression of sterol 27-hydroxylase has not been assessed.

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1. Abbreviations used in this paper: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PC, pericentral; PP, periportal.

One of the major regulatory processes affecting bile acid biosynthesis is bile acid-induced feedback inhibition, which is exerted at the level of cholesterol 7 $\alpha$ -hydroxylase by the flux of bile acids returning to the liver via portal blood (9). Bile acids are taken up efficiently by the periportal hepatocytes (15–19), thereby creating decreasing concentration gradients along the sinusoids (20). Consequently, periportal hepatocytes are exposed to a sixfold higher concentration of bile acids, as compared with pericentral cells (15, 16). In line with this, a concentration-dependent and direct downregulation of bile acid synthesis, at the level of cholesterol 7 $\alpha$ -hydroxylase, was found in vitro when cultured pig (21) and rat (22, 23) hepatocytes were incubated with bile acids. In a recent paper, our group demonstrated that sterol 27-hydroxylase is regulated in parallel with cholesterol 7 $\alpha$ -hydroxylase in cultured rat hepatocytes, resulting in coordinate downregulation of both enzymes at the mRNA and transcriptional level by similar bile acids. Thus it was postulated that efficient downregulation of bile acid synthesis by bile acids is accomplished by the coordinate regulation of both key enzymes (24).

In the present study, we have assessed the distribution patterns for both cholesterol 7 $\alpha$ -hydroxylase and sterol 27-hydroxylase within the liver lobulus. In addition, we established the molecular level at which these expression patterns are imposed, by measuring mRNA levels in freshly isolated periportal and pericentral hepatocytes, and transcriptional activity levels for these enzymes, by using nuclear run-off assays. Dynamic aspects of lobular distribution of these cholesterol-metabolizing enzymes and relationship of their expression to lobular bile acid concentrations were determined by treatment of rats with colestid. The bile acid sequestrant, like cholestyramine, has been shown to lead to upregulation of bile acid synthesis (25), as a result of lowering of the bile acid concentration in portal blood (26). Lobular distribution in control and stimulated rats was also assessed by in situ hybridization of sections of rat liver.

The current study shows heterogeneous distribution of both cholesterol 7 $\alpha$ -hydroxylase and sterol 27-hydroxylase. Heterogeneity resulted from coordinate differential transcriptional activity of both genes and, even more so, of steady-state mRNA levels for the enzymes, which reside primarily in the pericentral zone of the liver lobulus. Opposite expression was found for the HMG-CoA reductase gene. Treatment of rats with colestid led to a more overall recruitment of hepatocytes within the lobulus for bile acid synthetic capacity, resulting from increased expression of the cholesterol 7 $\alpha$ -hydroxylase and sterol 27-hydroxylase genes within a large part of the liver lobule.

## Methods

Materials used for isolation of rat hepatocytes, determination of cholesterol 7 $\alpha$ -hydroxylase and sterol 27-hydroxylase activity, and determination of mRNA and transcriptional activity levels have been described previously (22, 27–30).

**Animals.** Male Sprague-Dawley rats (200–280 g) were used for isolation of hepatocytes. Animals were kept in a strictly controlled 12-hr light/12-hr dark cycle (lights on from 06:00 to 18:00 h) on standard chow (Alma H 1003, Botzenhardt, Kempten, FRG) and tap water ad libitum. A separate group of animals were fed a similar diet supplemented with 5% (wt/wt) Colestid (Upjohn, Belgium) for a 7-d period before isolation (31). Time of isolation was between 08:00 and 09:00 h.

**Isolation of hepatocytes.** Total liver parenchymal cells were isolated by the two-step collagenase perfusion technique, modified as described by Gebhardt and Jung (28).

Table I. Activity of Marker Enzymes in Pericentral and Periportal Hepatocytes

Enzyme	Activity		PC/PP ratio
	Pericentral	Periportal	
	<i>nmol/min per mg of cell protein</i>		
Glutamine synthetase	632±167 (6)	13±6 (6)	48.6
Alanine aminotransferase	189±94 (6)	293±105 (6)	0.65
Pyruvate kinase	168±72 (5)	137±41 (4)	1.22

Pericentral and periportal hepatocytes were isolated as described in Methods. Purity of hepatocyte preparations was assessed by determination of activities for various marker enzymes. Data are expressed as absolute values  $\pm$ SD of enzyme activities, using hepatocytes from *n* rats, or as a ratio of PC/PP activity.

Periportal and pericentral subfractions of hepatocytes were isolated by the digitonin/collagenase perfusion technique, as described by Quistorff (32) and Lindros and Penttilä (33), with modifications described elsewhere (27). Viability, assessed by trypan blue exclusion, was > 90 and 80% for normal and isolated periportal and pericentral hepatocytes, respectively. Cell suspensions from digitonin/collagenase perfusions with a viability index of < 70% were discarded.

The efficiency of enrichment of periportal and pericentral hepatocytes was monitored by measurements of glutamine synthetase, alanine aminotransferase, and pyruvate kinase activity (Table I).

**Enzyme assays.** The activity of glutamine synthetase was determined by the glutamyltransferase assay with modifications reported previously (34). Alanine aminotransferase and pyruvate kinase were determined according to Bergmeyer (35). Cholesterol 7 $\alpha$ -hydroxylase and sterol 27-hydroxylase were assessed using homogenates as described in detail elsewhere (14, 29, 30). Protein and cholesterol were assayed according to methods previously described (30).

**RNA isolation, blotting, and hybridization procedures.** Total RNA was isolated from whole livers or freshly isolated periportal and pericentral hepatocytes, and quantitation of RNA was performed as previously described (22). Probes used in hybridization experiments were labeled by the random-primer method (Mega-prime, Amersham, Buckinghamshire, United Kingdom.) to  $\sim 6 \times 10^8$  cpm per  $\mu$ g of DNA. After hybridization and washing, the filters were exposed to Hyperfilm MP (Amersham) together with an intensifying screen (Eastman Kodak Co., Rochester, NY) for 48–120 h at  $-80^\circ\text{C}$ . For quantitation of the relative amounts of mRNA, the autoradiographs were scanned using a chromatograph scanner, (model CS 910; Shimadzu Corp., Kyoto, Japan), and areas under the curves were integrated using a data processor (Shimadzu Corp.). The following DNA fragments were used as probes in hybridization experiments: a 1.6-kb PCR-synthesized fragment of rat cholesterol 7 $\alpha$ -hydroxylase cDNA, spanning the entire coding region as described in detail by Twisk et al. (22); a 1.6-kb HindIII–XbaI fragment of rat sterol 27-hydroxylase cDNA, kindly provided by Dr. Jerome Strauss (36) and isolated from a rat liver cDNA library using the rabbit sterol 27-hydroxylase cDNA, previously isolated by Andersson et al. (37), as a probe; a 700-bp EcoRI fragment of hamster lithocholic acid 6 $\beta$ -hydroxylase cDNA (38); a 773-bp HindIII fragment of hamster HMG-CoA reductase cDNA (39); a 1.5-kb PstI fragment of rat glutamine synthetase cDNA (40, 41); a 1.2-kb PstI fragment of hamster actin cDNA; and a 1.1-kb PstI fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The latter two probes served as an internal standard to correct for differences in the amount of total RNA applied onto the gel or filter.

**Nuclear run-off studies.** Nuclear run-on studies were conducted essentially as described by Twisk et al. (22). Whole livers, or freshly isolated periportal and pericentral hepatocytes, from control and colestid-treated rats served as material for the isolation of nuclei. For whole

liver preparations, livers were perfused with saline solution (0.9% NaCl), cut into small fragments, washed, and homogenized mechanically (10 strokes, 180 rpm) in NP-40 lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl<sub>2</sub>, 0.5% NP-40, 1 mM PMSF, 1 mM DTT), at 4°C. The resulting suspension was filtered (100 μm) before an additional homogenizing step. Alternatively, directly after isolation, periportal and pericentral hepatocytes were washed and resuspended in NP-40 lysis buffer. Both preparations were further homogenized, after being left on ice for 5 min, in an all-glass Dounce homogenizer with pestle B (Bellco Glass Inc., Vineland, NJ) for 15 strokes at 4°C. Resulting nuclei were centrifuged at 500 g and resuspended in NP-40 lysis buffer. Washing procedures in NP-40 lysis buffer were repeated until the nuclei were free of cellular debris. They were then taken up in glycerol storage buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 1 mM PMSF, 5 mM DTT), counted, and aliquoted at ~ 2 × 10<sup>7</sup>/500 μl before being frozen at -80°C.

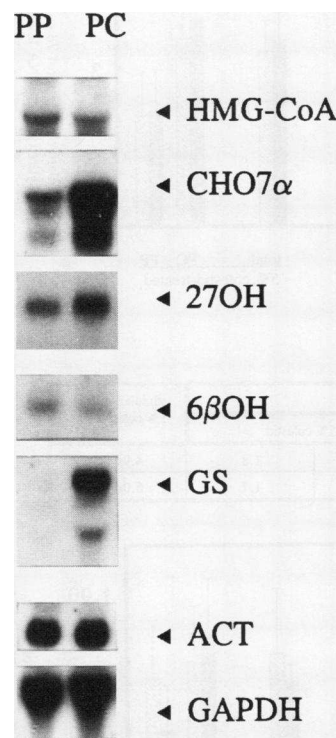
RNA labeling and isolation were performed as described (22). Target DNAs (5 μg of plasmid material containing cDNA sequences of rat cholesterol 7α-hydroxylase, rat sterol 27-hydroxylase, hamster HMG-CoA reductase, hamster lithocholic acid 6β-hydroxylase, hamster actin, and rat GAPDH, or the empty vector pUC19) were slot blotted onto strips of filter (Hybond-N<sup>+</sup>, Amersham) and cross-linked. The filters were hybridized with the <sup>32</sup>P-labeled RNA for 36 h, washed, and exposed to Hyperfilm MP for 2–5 d. Quantitation of relative amounts of mRNA synthesized was conducted using a Phosphorimager 400B (Molecular Dynamics, Sunnyvale, CA).

*In situ hybridization experiments.* Liver tissue was fixed in 4% formaldehyde solution and quickly frozen in liquid Freon 22, for preparation of sections (41). The in situ hybridization experiments were performed on closely adjacent sections to allow easy comparison of the patterns of hybridization. <sup>35</sup>S-labeled probes for in situ hybridization were prepared using the multiprime DNA labeling method, to a specific activity of 5 × 10<sup>8</sup> to 10 × 10<sup>8</sup> cpm per μg of DNA. Prehybridization treatments, hybridization, and autoradiography were performed precisely as described previously (41, 42). Negative controls included RNase-treated sections and hybridizations with an empty vector (pBR322).

## Results

*Heterogeneous distribution of cholesterol 7α-hydroxylase and sterol 27-hydroxylase.* Pericentral and periportal hepatocytes were isolated by digitonin/collagenase perfusion as described (43), and purity of the different preparations was determined in terms of enrichment of specific marker enzymes known to be differentially expressed. Table I shows strong predominant expression of glutamine synthetase in the pericentral fraction (the pericentral (PC)/periportal (PP) ratio being 48.6), in agreement with expression of this enzyme within the most distal pericentral hepatocytes surrounding the central venules of rat liver (43, 44). Other marker enzymes, alanine aminotransferase and pyruvate kinase, showed PC/PP ratios of 0.65 and 1.22, respectively, in agreement with data reported by others (33). Taken together, the perfusion technique allows for a good separation of hepatocytes into a pericentral and periportal fraction, keeping differential expression of several known marker enzymes intact.

Northern blotting of total RNA isolated from periportal and pericentral hepatocytes (Fig. 1) shows the typical expression pattern for cholesterol 7α-hydroxylase in both cell preparations (mRNAs of 2.1, 3.6, and 4.0 kb, as previously reported [22, 45, 46]). Clearly, expression of cholesterol 7α-hydroxylase mRNA is particularly strong in the pericentral area, as is that of glutamine synthetase (two mRNAs of 1.6 and 2.8 kb, as described [47]), which was assessed for reasons of comparison. The latter is in agreement with exclusive expression of both

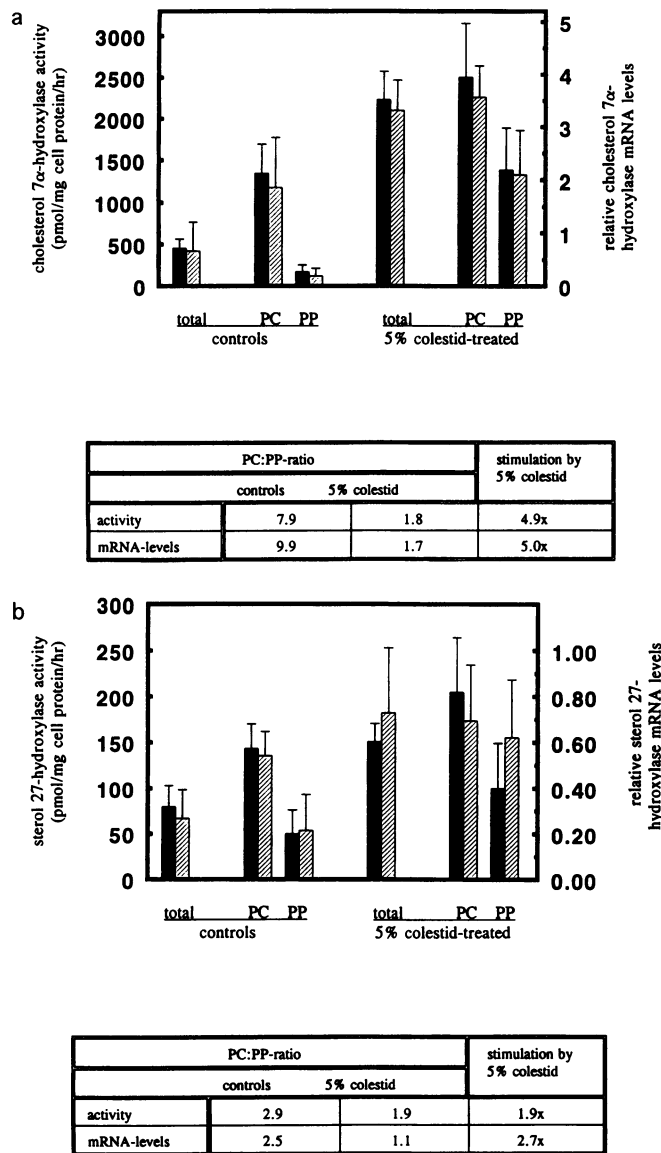


*Figure 1.* Distribution patterns for cholesterol 7α-hydroxylase and sterol 27-hydroxylase mRNA in periportal and pericentral hepatocytes. Periportal (PP) and pericentral (PC) hepatocytes were isolated by digitonin/collagenase perfusion, as described in Methods. Immediately after isolation of hepatocytes, total RNA was isolated for mRNA analysis. 10 μg of total RNA was electrophoresed in a 0.8% agarose/1 M formaldehyde gel, transferred to Hybond-N<sup>+</sup>, and subsequently hybridized with <sup>32</sup>P-labeled cDNA probes for HMG-CoA reductase (HMG-CoA), cholesterol 7α-hydroxylase (CHO7α), sterol 27-hydroxylase (27OH), lithocholic acid 6β-hydroxylase (6βOH), glutamine synthetase (GS), β-actin (ACT), and GAPDH. The latter two served as an internal standard. Resulting filters were subjected to autoradiography for 2–5 d, or for 2–3 wk for detection of HMG-CoA reductase mRNA.

mRNA and protein (41, 47) in a very limited fraction of pericentral cells. Sterol 27-hydroxylase (2.4 kb in rat liver) (24, 36) is expressed less abundantly in rat liver, as compared with the messengers previously described. Nevertheless, this mRNA was also localized predominantly pericentrally. Fig. 2 summarizes the distribution of cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity and mRNA levels (the latter relative to β-actin mRNA). β-Actin mRNA was used as an internal standard and did not vary between different cell preparations or from control (Fig. 1) or colestid-treated rats (data not shown). Both cholesterol 7α-hydroxylase activity and mRNA levels are predominant in hepatocytes from the pericentral area, showing respective PC/PP ratios of 7.9 and 9.9 (Fig. 2 a). For sterol 27-hydroxylase, heterogeneity of expression is less extreme, showing a 2.9- and 2.5-fold higher activity and mRNA level, respectively, within the pericentral hepatocytes (Fig. 2 b).

In view of reports on the periportal localization of HMG-CoA reductase, a key enzyme in the cholesterol biosynthetic route (7, 8), it was of interest to assess the relative mRNA levels for this enzyme within the liver lobulus as well and thus obtain an additional internal control for the identity of periportal hepatocytes. HMG-CoA reductase mRNA levels were very low, both in periportal and pericentral hepatocytes, as indicated by the longer exposure time required (legend to Fig. 1). Low levels of HMG-CoA reductase mRNA in livers of control rats were reported by others (48, 49) as well. Nevertheless, a mean PC/PP ratio of 0.5 (n = 3) was detected, in agreement with positive immunohistochemical staining for the HMG-CoA reductase protein of hepatocytes located in the periportal zone (7, 8).

Expression of lithocholic acid 6β-hydroxylase, which is primarily involved in metabolism of secondary bile acids returning to the liver via portal blood (i.e., lithocholic acid) (38), was



**Figure 2.** Distribution patterns for cholesterol 7 $\alpha$ -hydroxylase and sterol 27-hydroxylase activity and mRNA levels in isolated pericentral and periportal hepatocytes from control and colestid-treated rats. Enzyme activity for cholesterol 7 $\alpha$ -hydroxylase and sterol 27-hydroxylase in isolated pericentral and periportal hepatocytes was assessed as described in Methods. RNA samples were prepared and mRNA analysis was performed as described in Fig. 1. Rats used for hepatocyte preparations were fed either normal chow or a diet supplemented with 5% colestid (wt/wt). Values are expressed in terms of absolute enzyme activity (solid bars), or in arbitrary units of mRNA relative to expression of  $\beta$ -actin (hatched bars), and are mean  $\pm$  SD using hepatocytes from 4–7 rats. PC/PP ratios are indicated at the bottom of the figure, as is the extent of stimulation in total hepatocyte preparations by the colestid treatment. (a) Cholesterol 7 $\alpha$ -hydroxylase; (b) sterol 27-hydroxylase.

found preferentially in the periportal zone (Fig. 1; PC/PP ratio of 0.4,  $n = 3$ ). The latter indicates that not all mRNAs of enzymes involved in bile acid biosynthesis are similarly localized.

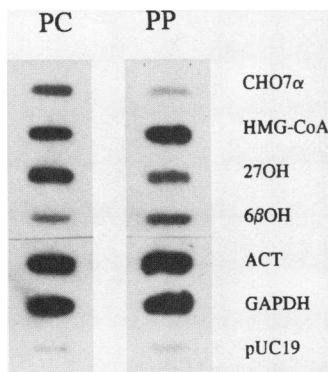
*Effect of colestid treatment of rats on the heterogeneity of mRNA patterns for different enzymes.* Rats were treated with 5% colestid for 7 d before isolation of hepatocytes. The bile

acid-sequestering property of this agent, like that of cholestyramine, leads to upregulation of bile acid synthesis in man (25) and rat (11, 12), as a result of diminished bile acid concentrations in portal blood (26). Assessment of activity for marker enzymes within different hepatocyte preparations from these rats revealed PC/PP ratios similar to those found in control rats (data not shown). As shown in Fig. 2 a, feeding rats a colestid-supplemented diet resulted in overall stimulation of both enzyme activity and mRNA levels for cholesterol 7 $\alpha$ -hydroxylase. The strong increase in cholesterol 7 $\alpha$ -hydroxylase activity (4.9-fold) and mRNA (5.0-fold) in whole liver preparations of stimulated rats is due mainly to stimulation of both parameters in the periportal hepatocytes. Levels for cholesterol 7 $\alpha$ -hydroxylase activity within this zone rose from  $170 \pm 81$  to  $1,386 \pm 504$  pmol/h per mg of cell protein (8.2-fold), as did mRNA levels for this enzyme (11.1-fold), whereas both parameters were stimulated only 1.9-fold in the pericentral hepatocytes. Consequently, PC/PP ratios for cholesterol 7 $\alpha$ -hydroxylase activity and mRNA were lowered to 1.8 and 1.7, respectively, in livers of stimulated rats.

Sterol 27-hydroxylase activity and mRNA (Fig. 2 b) were also stimulated in colestid-treated rats (1.9- and 2.7-fold, respectively, as compared with control rats), though less markedly. Sterol 27-hydroxylase mRNA was specifically upregulated in the portal zone (2.9-fold), thereby lowering the PC/PP ratio from 2.5 in control rats to 1.1 in colestid-treated animals. Sterol 27-hydroxylase activity was mildly stimulated in hepatocytes from both zones, particularly in the portal fraction (2-fold).

HMG-CoA reductase mRNA levels were also increased in colestid-treated rats (4.5-fold; data not shown), in agreement with upregulation of mRNA (48, 49) and activity levels (49, 50) for this enzyme by bile acid sequestrants. In contrast to cholesterol 7 $\alpha$ -hydroxylase, however, this particular increase is not a result of overall expression of the messenger in livers from stimulated rats, but rather of selective upregulation in the portal zone. HMG-CoA reductase mRNA was stimulated 7.8-fold in the portal region and only 2.7-fold in the pericentral area. The PC/PP ratio for mRNA of this enzyme was hence lowered even further, from 0.5 in control livers to 0.1 in livers from colestid-treated rats.

*Transcriptional activity of the cholesterol 7 $\alpha$ -hydroxylase and sterol 27-hydroxylase genes in different zones of the liver lobulus.* Nuclei from freshly isolated pericentral and periportal hepatocytes were used in nuclear run-off assays. Fig. 3 shows a typical autoradiograph of a hybridization experiment in which  $^{32}$ P-labeled RNA from pericentral and periportal nuclei was hybridized to cDNAs specific for cholesterol 7 $\alpha$ -hydroxylase, HMG-CoA reductase, sterol 27-hydroxylase, and lithocholic acid 6 $\beta$ -hydroxylase. As internal standards, transcriptional activities of the  $\beta$ -actin and GAPDH genes were also assessed in these nuclear preparations. Although no difference was found in expression patterns of the  $\beta$ -actin and GAPDH genes, expression of the cholesterol 7 $\alpha$ -hydroxylase gene is clearly pericentral (PC/PP ratio of 4.4; Fig. 4 a). Similarly, transcriptional activity of the sterol 27-hydroxylase gene was found to be highest in the pericentral region (PC/PP ratio of 1.7). In contrast, HMG-CoA reductase gene expression was localized preferentially in periportal cells (PC/PP ratio of 0.7), in agreement with opposite lobular localization of mRNA for this enzyme. The overall transcriptional activity is high for HMG-CoA reductase, which reached levels somewhat lower than those of  $\beta$ -actin and GAPDH. This indicates that posttranscriptional pro-



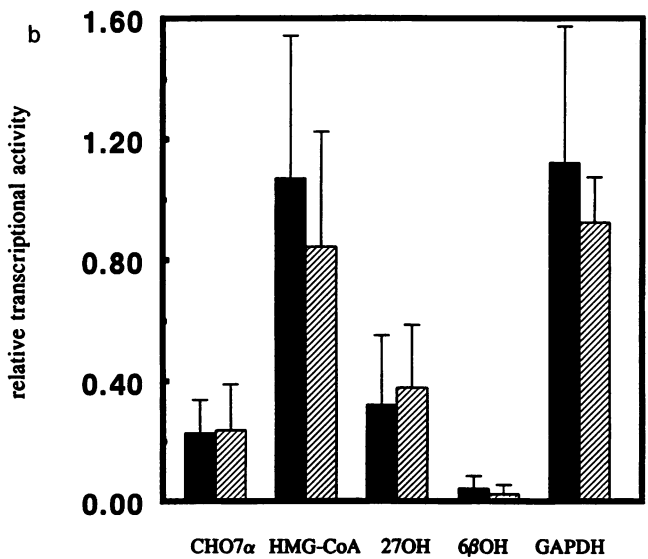
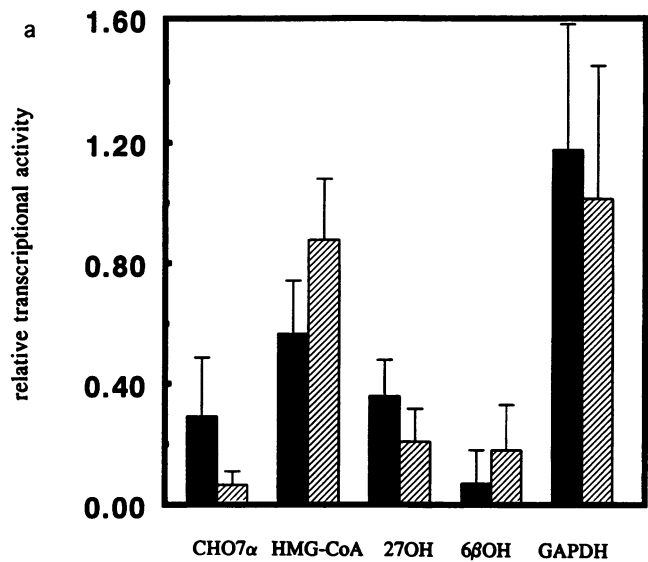
**Figure 3.** Transcriptional activity of the cholesterol 7 $\alpha$ -hydroxylase and sterol 27-hydroxylase genes in isolated pericentral and periportal hepatocytes. Nuclei were prepared from freshly isolated pericentral (PC) and periportal (PP) hepatocytes, as described in Methods. <sup>32</sup>P-labeled total RNA was synthesized in vitro using these nuclear preparations and hybridized to different cDNA probes. Resulting filters were subjected to autoradiography. Probes used were cDNAs for cholesterol 7 $\alpha$ -hydroxylase (CHO7 $\alpha$ ), HMG-CoA reductase (HMG-CoA), sterol 27-hydroxylase (27OH), lithocholic acid 6 $\beta$ -hydroxylase (6 $\beta$ OH),  $\beta$ -actin (ACT), and GAPDH. The latter two served as internal standards. Nonspecific hybridization was checked using an empty vector (pUC19).

cesses may be responsible for the low mRNA levels observed for this enzyme. Lithocholic acid 6 $\beta$ -hydroxylase gene expression was also localized predominantly periportally (PC/PP ratio of 0.4), in agreement with expression of mRNA levels for this enzyme (Fig. 1).

Treatment of rats with 5% colestid resulted in enhanced transcriptional activity in whole liver preparations of the cholesterol 7 $\alpha$ -hydroxylase (3.6-fold), sterol 27-hydroxylase (2.2-fold), and HMG-CoA reductase (5.5-fold) genes. In contrast, lithocholic acid 6 $\beta$ -hydroxylase gene transcription was downregulated (3.6-fold), whereas the housekeeping genes  $\beta$ -actin and GAPDH were not affected by colestid treatment. Analysis of gene expression in isolated pericentral and periportal cells from stimulated rats (Fig. 4b) showed marked expression of cholesterol 7 $\alpha$ -hydroxylase and sterol 27-hydroxylase genes over the entire portocentral axis. Upregulation of transcriptional activity in the pericentral zone was found only for the HMG-CoA reductase gene, whereas gene expression for lithocholic acid 6 $\beta$ -hydroxylase was specifically downregulated in the portal zone.

*In situ hybridization of livers from control and colestid-treated rats.* Fig. 5 shows heterogeneous expression of cholesterol 7 $\alpha$ -hydroxylase and sterol 27-hydroxylase mRNA, as detected by in situ hybridization of liver sections from control and colestid-treated rats. Cholesterol 7 $\alpha$ -hydroxylase (Fig. 5a) is abundantly and almost exclusively expressed in a limited fraction of hepatocytes surrounding the terminal venules. Particular pericentral expression was also detected for glutamine synthetase (Fig. 5c), as reported previously (41, 47), and was assessed for reasons of comparison and positive zonal identification. In situ hybridization of control livers with the rat sterol 27-hydroxylase cDNA probe also showed positive staining of pericentral hepatocytes only, though the staining was far less abundant and less discrete. This is in agreement with the mRNA analysis shown previously for this enzyme (Fig. 2b). Rather, a slight gradient of weak positive staining, declining toward the periportal zone, was found (Fig. 5b).

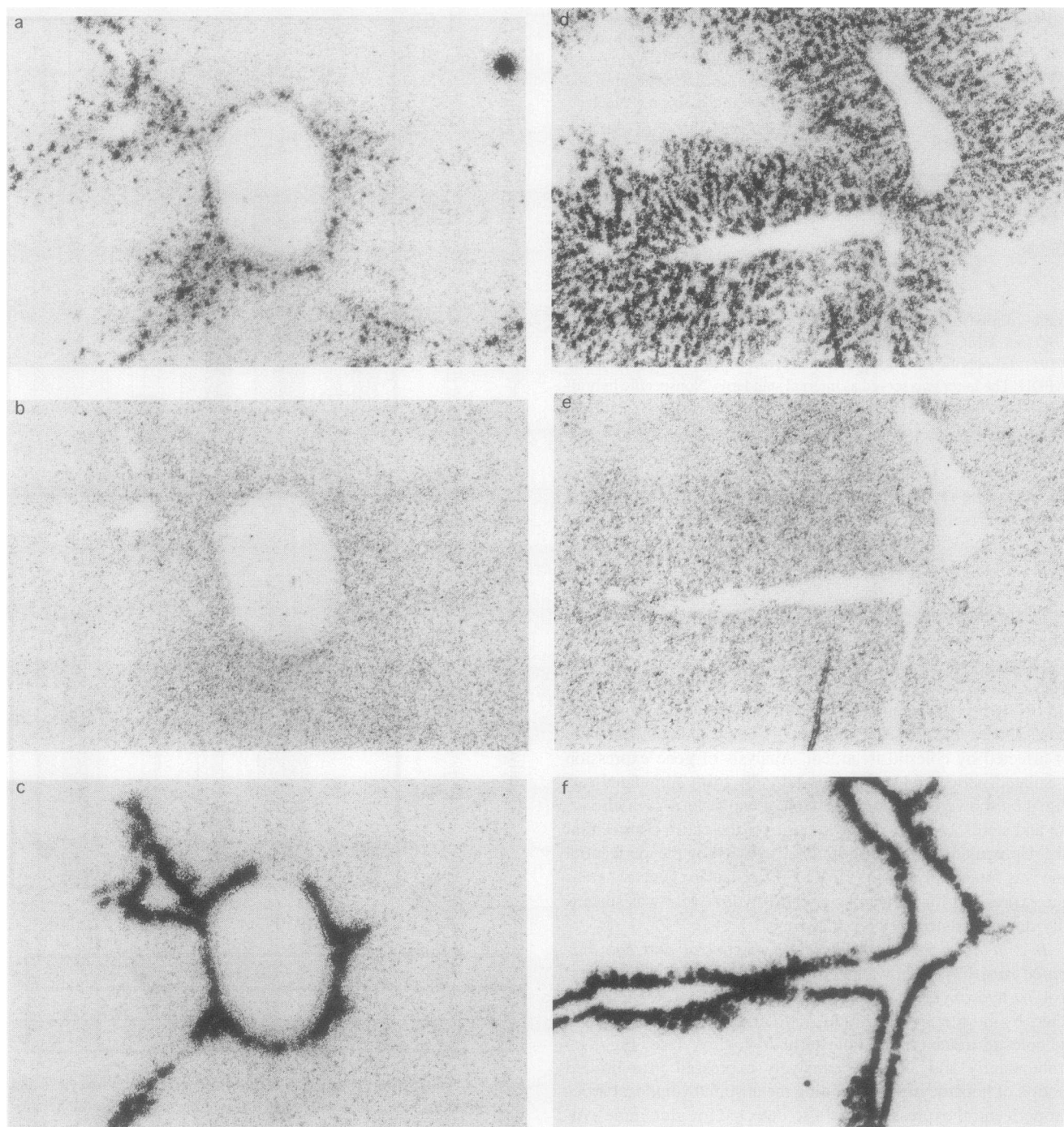
Treatment with colestid caused a more abundant expression of cholesterol 7 $\alpha$ -hydroxylase mRNA throughout a large section of the lobulus (Fig. 5d), concomitant with observed expression patterns of cholesterol 7 $\alpha$ -hydroxylase activity and mRNA. Sterol 27-hydroxylase mRNA showed a similar but less marked upregulation by colestid treatment, resulting in positive staining



PC:PP-ratio					
controls	4.4	0.7	1.7	0.4	1.2
5% colestid	1.0	1.3	0.9	1.8	1.2

**Figure 4.** Transcriptional activity of the cholesterol 7 $\alpha$ -hydroxylase and sterol 27-hydroxylase genes in isolated pericentral and periportal hepatocytes from control and colestid-treated rats. Nuclear preparations were prepared from control rats and from animals fed chow supplemented with 5% colestid. Run-off assays were performed as described in Fig. 3 and in Methods. Levels of transcriptional activity in the different hepatocyte subfractions were calculated relative to expression of  $\beta$ -actin, used as an internal standard, and are presented as a mean  $\pm$  SD using hepatocytes from 4–7 rats. (a and b) Freshly isolated pericentral (solid bars) and periportal (hatched bars) hepatocytes served as material for the isolation of nuclei, using control (a) and colestid-treated (b) rats. cDNA probes used were as described in Fig. 3. PC/PP ratios are indicated at the bottom of the figure.

of a large fraction of hepatocytes within each lobular unit (Fig. 5e). Glutamine synthetase showed no colestid-induced effect and remained pericentrally localized (Fig. 5f). The latter



**Figure 5.** Localization of cholesterol  $7\alpha$ -hydroxylase and sterol  $27$ -hydroxylase mRNA on serial liver sections by in situ hybridization. Liver sections were made from control (*a-c*) and colestid-treated (*d-f*) rats as described in Methods and were hybridized in situ with  $^{35}\text{S}$ -labeled probes for cholesterol  $7\alpha$ -hydroxylase (*a* and *d*), sterol  $27$ -hydroxylase (*b* and *e*), and glutamine synthetase (*c* and *f*). The latter was assessed as a positive identification of the pericentral zone. Sections depicted are of a central vein with surrounding hepatocytes.

agrees well with reported rigid heterogeneity of this enzyme in rat liver (51, 52).

### Discussion

The current study shows that key enzymes involved in bile acid biosynthesis, cholesterol  $7\alpha$ -hydroxylase and sterol  $27$ -hydroxylase, predominate in the pericentral area of the rat liver lobulus. Under normal feeding conditions, the distribution of

both enzymes is accomplished by parallel expression of mRNA levels and transcriptional activity of the corresponding genes. The localization is dynamic and responds to reduced portal bile acid concentrations after colestid treatment by changing distribution patterns for transcriptional activities, and particularly mRNA levels of these enzymes, to a more overall expression. This results in recruitment of a larger portion of hepatocytes within the liver lobulus for bile acid synthetic purposes.

*Parallel lobular distribution of cholesterol 7 $\alpha$ -hydroxylase and sterol 27-hydroxylase.* A strong preferential expression of cholesterol 7 $\alpha$ -hydroxylase activity, mRNA, and gene transcription was detected within the pericentral zone (PC/PP ratios of 7.9, 9.9, and 4.4, respectively). These results provide the molecular basis for the reported predominantly pericentral expression of bile acid synthesis and cholesterol 7 $\alpha$ -hydroxylase activity (11, 12). In addition, the present data show that enzyme activity and mRNA level for sterol 27-hydroxylase are also higher in the pericentral zone (PC/PP ratios of 2.9 and 2.5, respectively), as a result of a higher transcriptional activity of the corresponding gene (1.7-fold) in this area. The latter enzyme is involved in alternative routing to bile acids via initial 27-hydroxylation of cholesterol, a pathway that has been shown to contribute considerably to bile acid synthesis, both in vivo in man (13) and in cultured human and rat hepatocytes (14).

*Coordinate bile acid-induced feedback of bile acid synthetic enzymes.* Almost nothing is known about regulation of sterol 27-hydroxylase. It has been suggested that the enzyme is of minor importance for the regulation of bile acid synthesis and the composition of bile acids formed in rat (53). However, recent in vitro studies showed that sterol 27-hydroxylase may be regulated by factors known to also affect cholesterol 7 $\alpha$ -hydroxylase. Both enzymes were inhibited to a similar extent by bile acids (24) and insulin (54). Thus it is conceivable that the alternative pathway is also subject to regulation in vivo and during maintenance of cholesterol homeostasis. The colocalized expression of both cholesterol 7 $\alpha$ -hydroxylase and sterol 27-hydroxylase activities and mRNA levels, further confirmed by in situ hybridization experiments, strongly suggests the involvement of both enzymes in determining expression of total bile acid synthesis within the lobulus.

The data presented support the concept that bile acid synthesis in vivo is regulated by the flux of bile acids returning to the liver through enterohepatic circulation. Although in principle all hepatocytes have equal uptake capacity for bile acids (19, 55), the microanatomy of the liver lobule results in a lobular concentration gradient during blood flow through the liver (15, 16, 55). We have suggested that high bile acid concentrations are responsible for major downregulation of bile acid synthesis specifically in the portal area, whereas the route is hardly affected in the pericentral zone (11, 12, 56). A relationship between portal bile acid concentrations and bile acid biosynthetic capacity of hepatocytes has been assumed previously (57, 58). Further evidence for direct regulation of both cholesterol 7 $\alpha$ -hydroxylase and sterol 27-hydroxylase by bile acids was obtained recently with in vitro studies, which showed a concentration-dependent downregulation of enzyme activities and mRNA by bile acids in cultured rat hepatocytes manifest at the transcriptional level (24).

Treatment of rats with bile acid sequestrants has been shown to result in lowering of the bile acid gradient over the liver lobulus, as a consequence of diminished bile acid concentrations in portal blood (26). Subsequent upregulation of bile acid synthesis as a result of colestid treatment may be caused by a reduced downregulation within the lobulus (11, 12). In addition, as a result of diversion of bile acids in this way, loss of zonal heterogeneity of bile acid excretion and cell polarity, in terms of cellular translocation of bile acids, has been demonstrated (59). The current study shows that stimulation of bile acid synthesis by colestid treatment may result from derepres-

sion of transcriptional activity of the cholesterol 7 $\alpha$ -hydroxylase and sterol 27-hydroxylase genes and particularly of the corresponding mRNA levels in the portal zone. The latter results provide further evidence that bile acids are directly involved in inducing distribution patterns for cholesterol 7 $\alpha$ -hydroxylase and sterol 27-hydroxylase within the liver lobulus. Furthermore, the colestid treatment shows that heterogeneity for these enzymes is not rigid, but responds to changing metabolic requirements.

*The molecular level of regulation by bile acids.* The heterogeneous expression of mRNA levels for both enzymes is induced at the level of gene transcription. Nevertheless, although relative levels for sterol 27-hydroxylase mRNA and transcriptional activity within the lobulus are closely linked, the stronger PC/PP ratio for cholesterol 7 $\alpha$ -hydroxylase mRNA, as compared with transcriptional activity of the gene (PC/PP ratios of 9.9 and 4.4, respectively), suggests that ultimate expression of mRNA and activity for this enzyme may be regulated at multiple levels. The distinct expression of cholesterol 7 $\alpha$ -hydroxylase mRNA in only a few pericentral cells (Fig. 5 a), whereas transcriptional activity for this gene is low, but not absent in the periportal region, substantiates this view. This becomes even more apparent after colestid treatment, which results in upregulated levels of cholesterol 7 $\alpha$ -hydroxylase mRNA in both zones of the lobulus (11- and 2-fold, respectively, for PP and PC zones), whereas the transcriptional activity of the gene is affected only in the portal area, and to a modest degree (2-fold). In line with this thought, it has been suggested that stability of the cholesterol 7 $\alpha$ -hydroxylase messenger is an important determinant of steady-state mRNA levels for this enzyme with respect to regulation by bile acids (22, 60). AU-rich sequences present in the 3' noncoding region of cholesterol 7 $\alpha$ -hydroxylase mRNA may be involved in such a regulatory scheme (61).

mRNA levels and transcriptional activity analysis for lithocholic acid 6 $\beta$ -hydroxylase and HMG-CoA reductase showed that not all enzymes involved in maintenance of cholesterol homeostasis are colocalized. Portal expression of lithocholic acid 6 $\beta$ -hydroxylase is conceivable in view of involvement of this enzyme in metabolism of secondary bile acids. Periportal hepatocytes, subject to the highest bile acid concentrations, are expected to be most active in conversion of lithocholic acid to murideoxycholic acid. Additionally, it has been demonstrated that the enzyme is upregulated by feeding bile acids to hamsters (38). In agreement with this finding, we have shown that expression of the enzyme can be downregulated at the level of transcription by diversion of bile acids from the circulation (3.6-fold in whole liver preparations; Fig. 4, a and b). Consequently, high expression of lithocholic acid 6 $\beta$ -hydroxylase in the portal area, where blood rich in bile acids enters the liver, may reflect a protective mechanism of the liver to minimize hepatotoxic effects.

*The link between cholesterol synthesis and bile acid formation.* Opposite lobular localization of mRNA and transcriptional activity for HMG-CoA reductase is interesting, since it poses questions regarding interregulation of cholesterol and bile acid synthetic routes and how each may contribute to liver cholesterol homeostasis. Controversy exists, specifically with regard to functional pools of cholesterol contributing to bile acid formation. It has been postulated that newly synthesized cholesterol is the preferred substrate for cholesterol 7 $\alpha$ -hydroxylase (62, 63). Based on the results presented in this and other reports



(7, 8, 11, 12), enzymes involved in de novo cholesterol synthesis and bile acid synthesis are strictly separated, and therefore newly synthesized cholesterol cannot be the preferred substrate under normal physiological circumstances. In line with this assumption, Robins et al. have shown that liver-synthesized cholesterol is preferentially secreted into bile without being metabolized (64). Furthermore, Scheibner et al. recently demonstrated that the bulk of bile acids synthesized in the first short period after bile duct ligation originate from preformed cholesterol (65).

Stimulation of de novo cholesterol synthesis in control rats by treatment with mevalonate did not result in an effect on cholesterol 7 $\alpha$ -hydroxylase, whereas HMG-CoA reductase activity was profoundly inhibited (66). Similarly, administration of HMG-CoA reductase inhibitor to hypercholesterolemic patients had no impact on synthesis of acidic sterols in bile (67), nor did administration to gallstone patients have an effect on cholesterol 7 $\alpha$ -hydroxylase activity (68). These data demonstrate that under normal circumstances, manipulation of the de novo cholesterol pool does not result in alteration of bile acid synthesis levels.

In this and previous studies (11, 12), we have shown that treatment of rats with colestid leads to expansion of bile acid synthetic capacity within the lobulus. These results indicate a direct linkage between cholesterol synthesis and bile acid synthesis, as a result of diversion of bile acids, and may explain the increased use of de novo cholesterol under these circumstances (63, 65, 69, 70). Interestingly, HMG-CoA reductase mRNA, though upregulated by colestid, remained predominantly periportal localized. In agreement with these findings, it has been shown that activity of HMG-CoA reductase remains periportal after treatment of rats with cholestyramine, but that expression of the enzyme up to the pericentral area is accomplished when this treatment is combined with mevinolin administration to rats (7, 8).

In conclusion, the present study demonstrates that heterogeneous localization of bile acid synthesis is accomplished by preferential transcriptional activity and mRNA levels of key enzymes for this route, cholesterol 7 $\alpha$ -hydroxylase and sterol 27-hydroxylase, in the pericentral zone of the liver lobulus. Colocalization of the two enzymes provides insights to how feedback regulation of bile acid synthesis by bile acids is achieved and how the total cholesterol pool within the liver is regulated to meet different metabolic demands. The concept of metabolic zonation provides the basis for this understanding and may shed further light on interregulation of pathways involved in maintenance of cholesterol homeostasis in the liver.

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